



(19)

Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 767 178 A1

(12)

## EUROPEAN PATENT APPLICATION

(43) Date of publication:  
09.04.1997 Bulletin 1997/15(51) Int Cl. 6: C07K 14/52, A61K 38/19,  
C12P 21/02

(21) Application number: 96306997.6

(22) Date of filing: 26.09.1996

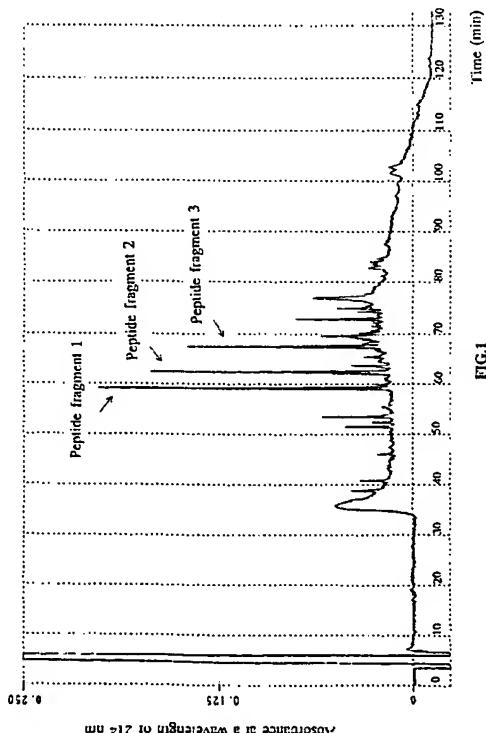
(84) Designated Contracting States:  
BE CH DE DK ES FR GB IT LI NL SE

- Nukada, Yoshiyuki  
Okayama-shi, Okayama (JP)
- Fujii, Mitsukiyo  
Okayama-shi, Okayama (JP)
- Tanimoto, Tadao  
Okayama-shi, Okayama (JP)
- Kurimoto, Masashi  
Okayama-shi, Okayama (JP)

(30) Priority: 26.09.1995 JP 270725/95  
29.02.1996 JP 67434/96  
20.09.1996 JP 269105/96(71) Applicant: KABUSHIKI KAISHA HAYASHIBARA  
SEIBUTSU KAGAKU KENKYUJO  
Okayama-shi Okayama (JP)(74) Representative: Daniels, Jeffrey Nicholas et al  
Page White & Farrer  
54 Doughty Street  
London WC1N 2LS (GB)(72) Inventors:  
• Akita, Kenji  
Okayama-shi, Okayama (JP)

## (54) Protein which induces interferon-gamma production by immunocompetent cell

(57) A protein of human cell origin, which induces the IFN- $\gamma$  production by immunocompetent cells and has the amino acid sequence of SEQ ID NO:1 near or at the N-terminus. It can be produced from human cells such as lymphoblasts, lymphocytes, monoblasts, monocytes, myeloblasts, myelocytes, granulocytes and macrophages, and used for preventing and/or treating IFN- $\gamma$  susceptible diseases.



**Description**

The present invention relates to a novel protein which induces the interferon- $\gamma$  (hereinafter abbreviated as "IFN- $\gamma$ ") production by immunocompetent cells.

It is known that IFN- $\gamma$  is a protein which has antiviral-, antioncotic- and immunoregulatory-activities and is produced by immunocompetent cells that are stimulated with antigens or mitogens. Because of these biological activities, IFN- $\gamma$  has been expected for use as an antitumor agent since it was discovered, and studied energetically on clinical trials as a therapeutic agent for malignant tumors in general including brain tumors. IFN- $\gamma$  preparations commercially available now are roughly classified into two groups, i.e. one group of natural IFN- $\gamma$ s produced by immunocompetent cells and another group of recombinant IFN- $\gamma$ s produced by transformants obtained by introducing DNAs which encode natural IFN- $\gamma$ s into microorganisms of the species *Escherichia coli*. In the above clinical trials, one of these two groups of IFN- $\gamma$ s is administered to patients as an "exogenous IFN- $\gamma$ ".

Among these IFN- $\gamma$ s, natural IFN- $\gamma$ s are usually produced by culturing established immunocompetent cell lines in nutrient culture media admixed with IFN- $\gamma$  inducers to produce IFN- $\gamma$ s, and purifying the produced IFN- $\gamma$ s from the resulting cultures. It is known that IFN- $\gamma$  inducers greatly influences on the IFN- $\gamma$  yield, the facility of IFN- $\gamma$  purification, and the safety of final IFN- $\gamma$  preparations. Generally, mitogens such as concanavalin A (Con A), lentil lectin, pokeweed lectin, endotoxin and lipopolysaccharides can be used as IFN- $\gamma$  inducers. However, these mitogens have the following problems: (i) their molecules and qualities vary and change depending on their origins and purification methods, and (ii) preparations with a constant IFN- $\gamma$  inducibility are not readily prepared in a satisfactory yield. In addition, most of these mitogens might induce unfavorable side effects when administered to living bodies, and some of them might cause toxicity, so that it is substantially difficult to induce IFN- $\gamma$  production by directly administering IFN- $\gamma$  inducers to the living bodies.

The present invention was made based on a novel protein which induces the interferon- $\gamma$  production by immunocompetent cells. During the study of cytokines produced by mammalian cells, the present inventors noticed that the existence of a substance which induces IFN- $\gamma$  production in mouse liver cells which had been treated with a lipopolysaccharide and inactivated whole cells of *Corynebacterium*. They isolated the substance by many purification methods using column chromatography as a main technique and studied the properties and features, and have found that the reality is a protein having the following physicochemical properties:

- 30       (1) Molecular weight  
19,000 $\pm$ 5,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE);
- (2) Isoelectric point (pI)  
pI of 4.8 $\pm$ 1.0 on chromatofocusing;
- (3) Partial amino acid sequence  
Having the partial amino acid sequences of SEQ ID NOs:8 and 9; and
- (4) Biological activity  
Inducing the IFN- $\gamma$  production by immunocompetent cells.

The data concluded that the substance is novel because no protein with these physicochemical properties is known. The present inventors continued studying on mouse liver cells and have succeeded to isolate a DNA which encodes the protein. The inventors decoded the DNA and have found that it consists of 471 base pairs and encodes the amino acid sequence of SEQ ID NO:10 (where the symbol "Xaa" means "methionine" or "threonine").

Based on these findings, the present inventors further studied on human liver cells to obtain a DNA which encodes another novel substance that induces the IFN- $\gamma$  production by immunocompetent cells. They revealed that the reality is a polypeptide, then decoded the DNA and found that it has the amino acid sequence of SEQ ID NO:6 (where the symbol "Xaa" is "isoleucine" or "threonine"). They introduced the DNA into *Escherichia coli* to express the polypeptide and to produce it in the resulting culture in a satisfactorily high yield. These findings were disclosed in Japanese Patent Laid-Open Nos.27,189/96 and 193,098/96, applied by the present applicant. In Japanese Patent Application No. 78,357/95 applied by the applicant, the polypeptide is disclosed as an agent for susceptive diseases. Although biologically active proteins which are administered to humans after mixed with pharmaceuticals should be generally human cell origin, no human cell which produces such a polypeptide is reported.

In view of the foregoing, the present invention aims to provide a protein of human cell origin, which induces the IFN- $\gamma$  production by immunocompetent cells.

The present invention further aims to provide a process for producing the protein.  
The present invention yet further aims to provide the use of the protein as an agent for susceptive diseases.  
The first aim of the present invention may be attained by a protein of human cell origin which induces the IFN- $\gamma$  production by immunocompetent cells and has the amino acid sequence of SEQ ID NO:1.

The second aim of the present invention may be attained by a process for producing the protein by propagating

human cells which produce the protein, and collecting the protein from the propagated cells.

The third aim of the present invention may be attained by an agent for susceptive diseases, which contains the protein as an effective ingredient.

FIG. 1 is a peptide map of a protein in accordance with the present invention

5 The protein according to the present invention induces the IFN- $\gamma$  production by immunocompetent cells when allowed to act on the cells alone or together with an appropriate cofactor.

The protein is derived from human cells, and it can be readily prepared by the present process using human cells.

10 The agent for susceptive diseases according to the present invention induces the IFN- $\gamma$  production by immunocompetent cells in the human body when administered to humans, and exerts positive effects in the treatment and prevention of IFN- $\gamma$  susceptive diseases. When the protein augments the cytotoxicity of killer cells or induces the formation of killer cells, it exerts positive effects on inveterate diseases including malignant tumors.

15 The preferred embodiments according to the present invention will be described hereinafter. The wording "protein" as referred to in the present invention means polypeptides and glycoproteins in general which induce the IFN- $\gamma$  production by immunocompetent cells and have the amino acid sequence of SEQ ID NO:1. Depending on the types and propagation conditions of human cells, the protein may have the amino acid sequences of SEQ ID NOS:1 and 3 near or at the N- and C-termini, respectively. Occasionally the protein has the amino acid sequence of SEQ ID NO:6, as a complete amino acid sequence (where the symbol "Xaa" means "isoleucine" or "threonine"). Furthermore, the protein may include the amino acid sequence of SEQ ID NOS:4 and/or 5 as an internal fragment. The protein is detected as a protein band at a position corresponding to a molecular weight of 14,000-24,000 daltons, usually, 18,000-19,500 daltons when determined on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent. Depending on the types and propagating conditions of human cells, one or more amino acids may be added to the above N- and/or C-termini of SEQ ID NOS:1, 2 or 3, or one or more amino acids in these sequences may be replaced with other amino acid(s), provided that these additions or replacements do not substantially alter the ability of the protein to induce the IFN- $\gamma$  production. Any protein can be used in the present invention as long as it is derived from a human cell, as well as having either of these amino acid sequences and inducing the IFN- $\gamma$  production when acting on immunocompetent cells alone or together with an appropriate cofactor.

20 These proteins can be produced by the present process using human cells. Usually, the human cells used in the present invention include cell lines derived from human hematopoietic cells such as lymphoblasts, lymphocytes, monoblasts, monocytes, myeloblasts, myelocytes, granulocytes and macrophages. Examples of these cell lines are lymphomas and leukemias such as myelocytic leukemia, promyelocytic leukemia, adult T-cell leukemia, and hairy cell leukemia, specifically, HBL-38 cell, HL-60 cell (ATCC CCL240), K-562 (ATCC CCL243), KG-1 cell (ATCC CCL246), Mo cell (ATCC CRL8066), THP-1 cell (ATCC TIB202), and U-937 cell (ATCC CRL1593) as reported by Jun MINOWADA in "Cancer Review", Vol. 10, pp.1-18 (1988), and A-253 cell (ATCC HTB41), an epidermoid carcinoma, submaxillary gland, human. Mutants of these cell lines can be also used in the present invention. Because these cell lines readily proliferate and more produce the present protein, they can be advantageously used in the present invention. Especially, epidermoid carcinoma cell lines such as A-253 cell, and human myelomonocytic cell lines such as HBL-38 cell, HL-60 cell, KG-1 cell, THP-1 cell, and U-937 cell have an extremely high productivity of the present protein and are most satisfactorily used in the present invention.

25 In the present process, the above human cells are first allowed to propagate, then the present protein is collected from the propagated cells. The method used to propagate these human cells in the present invention is not specifically restricted, and any conventional *in vivo* or *in vitro* propagation method can be used. The *in vivo* propagation method means a method to propagate cells using nutrient culture media, which comprises suspending human cells in RPMI 1640 medium, MEM medium and DEM medium, which are used conventionally to propagate animal cells in this field, supplemented with 0.3-30 w/v % of fetal bovine serum to give a cell density of about  $1 \times 10^4$ - $1 \times 10^7$  cells/ml, preferably, about  $1 \times 10^5$ - $1 \times 10^6$  cells/ml, and culturing these cells at a temperature of 36-38°C, preferably, a temperature of about 37°C and at a pH of 7-8, preferably, a pH of 7.2-7.4, for about 1-7 days while replacing these media with fresh ones. Thereafter, the propagated cells were separated from the cultures to obtain the objective protein. Depending on the types and culture conditions of human cells, some cells extracellularly excrete the present protein while culturing. When coexisted in culture media inducers such as mitogens and/or IFN- $\gamma$ s which induce the production of the present protein by the human cells, most of or all of the protein may be produced extracellularly. In this case, the protein can be collected from the culture supernatants.

30 The *in vivo* propagation method for human cells using warm-blooded animals excluding human comprises injecting to suppress the immunoreaction of the animals antilymphocyte antibodies derived from rabbits into rodents such as new born mice, nude mice, rats, nude rats, guinea pigs, and hamsters, injecting subcutaneously or intraperitoneally about  $1 \times 10^5$ - $1 \times 10^8$  cells/animal of the human cells into the animals or placing the human cells in diffusion chambers embedded in or out of the animals' body while allowing the animals' body fluid to circulate in the chambers, and feeding the animals by conventional methods for about 2-10 weeks. During the feeding, the human cells propagate while receiving the animals' body fluid. The propagated human cells are collected in the form of a tumor mass, ascites or

cell suspension. If necessary, the objective protein is collected after suspending and washing these human cells in and with an appropriate solvent. The *in vivo* propagation method has a merit that as compared with the *in vitro* propagation method it yields the present protein at a less labor cost and time and in a satisfactorily high yield. The *in vivo* propagation method is disclosed, for example, in Japanese Patent Publication No.54,158/81.

5 To collect the present protein from the propagated cells, these cells are disrupted by ultrasonic before or after separating the objective protein from the cultures, homogenizing, freezing and thawing, or by soaking these cells in considerably-low osmotic solvents, then the protein is collected from the resulting cell debris or from a mixture of cell debris and culture supernatant. To collect the protein from the cell debris or the mixture, the cell debris or the mixture can be subjected directly or after incubation at about 37°C for 1-24 hours to the following conventional methods for purifying biologically active substances in this field: salting out, dialysis, filtration, concentration, preparatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, adsorption chromatography, affinity chromatography, chromatofocusing, gel electrophoresis and/or isoelectrophoresis. Two or more of these conventional methods can be selectively used in combination. The collected protein can be concentrated and/or lyophilized into a liquid or solid form to meet to final use. The monoclonal antibody as disclosed in Japanese Patent Application No.58,240/95 applied by the present applicant is advantageously used to purify the present protein. Immunoaffinity chromatography using the monoclonal antibody yields the highest possible purity of the protein at the lowest cost and labor.

10 As is described above, the protein according to the present invention has a property of inducing the IFN- $\gamma$  production by immunocompetent cells. Thus it can be satisfactorily used as an inducer for IFN- $\gamma$  production by cell culture methods and used in the treatment and prevention of IFN- $\gamma$  susceptive diseases including viral diseases such as AIDS and condyloma acuminatum; malignant tumors such as malignant nephroma, granuloma, mycosis fungoides, and brain tumor; and immunopathies such as articular rheumatism and allergosis.

15 The present protein is usually added to nutrient culture media for IFN- $\gamma$  production by culturing immunocompetent cells or administering to humans to treat and/or prevent IFN- $\gamma$  susceptive diseases. In the former case, leukocytes separated from mammalian peripheral blood and established cell lines of immunocompetent cells such as HBL-38 cell, Mo cell (ATCC CRL8066), Jurkat cell (ATCC CRL8163), HuT78 cell (ATCC TIB161), EL4 cell (ATCC TIB39), L12-R4 cell, and mutants thereof are suspended in culture media containing about 0.1-1,000 ng/ml of the present protein, preferably, about 1-100 ng/ml of the protein. If necessary, these cells are cultured in nutrient culture media supplemented with T-cell stimulants such as mitogen, interleukin 2, and anti-CD3 antibody for about 1-100 hours in conventional manner while replacing the culture media with fresh ones. From the resulting cultures the present protein can be collected by one or more conventional methods used to purify IFN- $\gamma$  such as salting out, dialysis, filtration, concentration, preparatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, adsorption chromatography, affinity chromatography, chromatofocusing, gel electrophoresis and isoelectrophoresis.

20 Because the present protein induces the IFN- $\gamma$  production by human immunocompetent cells, agents for susceptive diseases containing the protein as an effective ingredient stimulate the human immunocompetent cells to produce IFN- $\gamma$  by administering to humans, and exert positive effects on the treatment and/or the prevention of IFN- $\gamma$  susceptive diseases. Killer cells participate in the treatment and/or the prevention of susceptive diseases when the present protein induces the IFN- $\gamma$  production by immunocompetent cells, accelerates the cytotoxicity of killer cells such as cytotoxic T-cells and lymphokine activating killer cells including NK- and LAK-cells, and induces the formation of killer cells similarly as the proteins in the later described Experiments and Examples. The wording "susceptive diseases" as referred to in the present invention means diseases in general including IFN- $\gamma$  susceptive diseases, which can be treated and/or prevented by IFN- $\gamma$ s and/or killer cells: For example, viral diseases such as hepatitis, herpes, condyloma acuminatum, and AIDS; infectious diseases such as candidiasis, malaria, cryptococcosis, and *Yersinia*; malignant solid tumors such as malignant tumor, mycosis fungoides, and chronic granulomatous disease; hematopoietic malignant tumors such as adult T-cell leukemia, chronic myelocytic leukemia, and malignant tumor; and immunopathies such as allergosis and rheumatism. When used with interleukin 3, the present protein positively effects on the complete cure or the remission of leukopenia and thrombocytopenia induced by radio- and chemotherapies to treat leukemia, myeloma, and malignant tumors.

25 The present agent for susceptive diseases is widely used in the treatment and/or the prevention of the above susceptive diseases as an antitumor agent, antiviral agent, antiseptic, immunotherapeutic agent, platelet-increasing agent, or leukocyte-increasing agent. Depending on the type of agent and the symptom of susceptive diseases to be treated, the present agent is generally processed into a liquid, paste or solid form which contains 0.000001-100 w/w %, preferably, 0.0001-0.1 w/w % of the protein, on a dry solid basis (d.s.b.).

30 The present agent can be used intact or processed into compositions by mixing with physiologically-acceptable carriers, adjuvants, excipients, diluents and/or stabilizers, and, if necessary, further mixing with one or more other biologically-active substances such as interferon- $\alpha$ , interferon- $\beta$ , interleukin 2, interleukin 3, interleukin 12, TNF- $\alpha$ , TNF- $\beta$ , carboquone, cyclophosphamide, aclarubicin, thiotepa, busulfan, ancitabine, cytarabine, 5-fluorouracil, 5-fluoro-

1-(tetrahydro-2-furyl)uracil, methotrexate, actinomycin D, chromomycin A<sub>3</sub>, daunorubicin, doxorubicin, bleomycin, mitomycin C, vincristine, vinblastine, L-asparaginase, radio gold colloidal, Krestin®, picibanil, lentinan, and Maruyama vaccine. Among these combinations, a combination of the present protein and interleukin 2 is especially useful because interleukin 2 acts as a cofactor for the protein when the protein induces the IFN-γ production by immunocompetent cells. Another combination of the protein and a natural or recombinant human interleukin 2 induces a relatively high level of IFN-γ production with only a small amount of the protein which does not substantially induce the IFN-γ production by immunocompetent cells. While a combination of the protein and interleukin 12 induces a greater level of IFN-γ production which could not be readily attained by them each. Because the present protein increases the activity of interleukin 12 to inhibit the production of immunoglobulin E antibody in the human body, the protein is advantageously used as an agent for immunopathies such as atopic diseases including atopic asthma, atopic bronchial asthma, hay fever, allergic rhinitis, atopic dermatitis, angioedema, and atopic digestive system's disorder. Occasionally a relatively small amount of interleukin 12 exists in humans. In this case, a sole administration of the protein to humans can attain the desired effect.

The form of the present agent for susceptive diseases includes those in a unit dose form which means a physically formulated medicament suitable for administration and contains the protein in an amount from 1/40 to several folds, i.e. up to 4 folds of a dosage. Examples of these are injections, liquids, powders, granules, tablets, capsules, sublinguals, ophthalmic solutions, nasal drops, and suppositories.

The present agent can be orally or parenterally administered to patients, and as described below it can be used to activate antitumor cells *in vitro*. In both administrations, the agent exerts a satisfactory effect in the treatment and/or the prevention of susceptive diseases. Varied depending on the types of susceptive diseases and the symptoms of patients before and after the administration, the agent is orally administered to them or parenterally administered to their intradermal- and subcutaneous-tissues, muscles, and veins at a dose of about 0.1 µg to 50 mg per shot, preferably, about one µg to one mg per shot, 1-4 times/day or 1-5 times/week, for one day to one year.

The present agent can be also used in so called "**antitumor immunotherapy**" using interleukin 2. Generally, the antitumor immunotherapy is roughly classified into (i) a method for directly administering interleukin 2 to patients with malignant tumors, and (ii) a method for introducing antitumor cells which are previously activated *in vitro* by interleukin 2, i.e. an adoptive immunotherapy. The present protein significantly enhances the above immunotherapeutic effect by interleukin 2 when used in combination. In the method (i), the protein is administered to patients in an amount of about 0.1 µg/shot/adult to one mg/shot/adult at 1-10 times before the administration of interleukin 2 or at the same time. The dose of interleukin 2 is generally about 10,000-1,000,000 units/shot/adult, though it varies depending on the types of malignant tumors, patients' symptoms, and the dose of the present protein. In the method (ii), mononuclear cells and lymphocytes, collected from patients with malignant tumors, are cultured in the presence of interleukin 2 and about 0.1 ng to one µg of the protein per  $1 \times 10^6$  cells of the blood cells. After culturing for a prescribed period of time, NK cells or LAK cells are collected from the culture and introduced into the same patients. Diseases which can be treated by the present antitumor immunotherapy are, for example, hematopoietic malignant tumors such as leukemia and malignant lymphoma, and solid malignant tumors such as colonic cancer, rectal cancer, large intestinal cancer, gastric cancer, thyroid carcinoma, cancer of the tongue, bladder carcinoma, choriocarcinoma, hepatoma, prostatic cancer, carcinoma uteri, laryngeal, lung cancer, breast cancer, malignant melanoma, Kaposi's sarcoma, cerebral tumor, neuroblastoma, tumor of the ovary, testicular tumor, osteosarcoma, cancer of the pancreas, renal cancer, hypernephroma, and hemangiendothelioma.

The following experiments explain the present protein:

#### Experiment 1

##### Preparation of protein

New born hamsters were suppressed their immunoreaction in conventional manner by injecting a rabbit antiserum to hamster antithymus into the hamsters, transplanted to their dorsal subcutaneous tissues with about  $5 \times 10^5$  cells/hamster of THP-1 cells (ATCC TIB202), a myelomonocytic cell line of a human acute monocytic leukemia, and fed for 3 weeks in conventional manner. Tumor masses formed in their subcutaneous tissues, about 15 g weight per hamster, were extracted, dispersed in conventional manner in physiological saline, and washed with phosphate buffered saline (hereinafter abbreviated as "PBS").

The propagated cells thus obtained were washed with 10-fold volumes of cold 20 mM Hepes buffer (pH 7.4) containing 10 mM potassium chloride, 1.5 mM magnesium chloride, and 0.1 mM disodium ethylenediaminetetraacetate, allowed to stand in 3-fold volumes of a fresh preparation of the same buffer under ice-chilled conditions, freezed at -80°C, and thawed to disrupt the cells. The disrupted cells were centrifuged to obtain a supernatant which was then fed to a column packed with "**DEAE-SEPHAROSE**", a gel for ion-exchange column chromatography commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been previously equilibrated with 10 mM phosphate

buffer (pH 6.6), followed by washing the column with 10 mM phosphate buffer (pH 6.6), feeding to the column with a gradient buffer of sodium chloride which increases stepwisely from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.6), and collecting a fraction eluted at about 0.2 M sodium chloride.

5 The fraction was dialyzed against 10 mM phosphate buffer (pH 6.8) and fed to a column packed with "DEAE 5PW", a gel for ion-exchange chromatography commercialized by Tosoh Corporation, Tokyo, Japan, followed by feeding to the column a gradient buffer of sodium chloride which increases stepwisely from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.8), and collecting fractions eluted at about 0.2-0.3 M sodium chloride.

10 The resulting fractions were pooled, then dialyzed against PBS, fed to a plastic cylindrical column packed with a gel for immunoaffinity chromatography using a monoclonal antibody which had been prepared according to the method as disclosed in Japanese Patent Application No.58,240/95 applied by the present applicant, and washed with PBS. The column was fed with 100 mM glycine-HCl buffer (pH 2.5) to collect from the eluate fractions containing a protein which induces the IFN- $\gamma$  production by immunocompetent cells. These fractions were pooled, dialyzed against sterile distilled water, concentrated with a membrane filter, and lyophilized to obtain a purified solid protein in a yield of about 50 ng per hamster.

15

### Experiment 2

#### Molecular weight

20 In accordance with the method reported by U. K. Laemmli in *Nature*, Vol.227, pp.680-685 (1970), a purified protein prepared by the method in Experiment 1 was electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) in the presence of 2 w/v % dithiothreitol, resulting in a main protein band with an IFN- $\gamma$  inducibility at a position corresponding to about 18,000-19,500 daltons. The marker proteins used in this experiment were bovine serum albumin (MW=67,000 daltons), ovalbumin (MW=45,000 daltons), carbonic anhydrase (MW=30,000 daltons), soy bean trypsin inhibitor (MW=20,100 daltons), and  $\alpha$ -lactalbumin (MW=14,400 daltons).

25

### Experiment 3

30

#### Amino acid sequence and peptide mapping near at the N-terminus

#### Experiment 3-1

#### Amino acid sequence near at the N-terminus

35

The purified protein in Experiment 1 was analyzed on "MODEL 473A", a protein sequencer commercialized by Perkin-Elmer Corp., Instrument Div., Norwalk, USA, and revealed that it has the amino acid sequence of SEQ ID NO: 1, particularly, SEQ ID NO:2.

40

#### Experiment 3-2

#### Peptide mapping

45

A purified protein obtained by the method in Experiment 1 was dissolved in an adequate amount of sterile distilled water, and the solution was fed to a column packed with "ASAHPAK® C4P-50 4E", a gel for high-performance liquid chromatography (HPLC) commercialized by Showa Denko, K.K., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetic acid solution, followed by washing the column with 0.1 v/v % aqueous trifluoroacetic acid solution and feeding to the column a linear gradient solution of acetonitrile increasing from 0 v/v % to 90 v/v % in a mixture solution of trifluoroacetic acid and acetonitrile at a flow rate of 60 ml/hour. Fractions containing a protein which induces the IFN- $\gamma$  production by immunocompetent cells were collected from the eluted fractions, pooled, neutralized with 1 M aqueous tris solution (pH 11.2), and concentrated in conventional manner. To 50 mM Tris-HCl buffer (pH 8.5), dissolving an adequate amount of clostripain commercialized by Sigma Chemical Company, St. Louis, Missouri, USA, was added the protein in an amount of about 50 folds of the clostripain by molar ratio while removing acetonitrile, and the resulting mixture was allowed to react at a pH of 8-9 and at 37°C for 12 hours to obtain a reaction mixture containing fragments of the protein.

50

55 The reaction mixture was fed to a column packed with "ODS-120T", a gel for HPLC commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetic acid solution, followed by washing the column with 0.1 v/v % aqueous trifluoroacetic acid solution and feeding to the column a linear gradient solution of acetonitrile increasing from 0 v/v % to 70 v/v % in a mixture solution of trifluoroacetic acid, acetonitrile

and water where the concentration of trifluoroacetic acid was 0.09 v/v % at a flow rate of 30 ml/hour while monitoring the absorption level of the peptide, i.e. the concentration of the peptide, at a wave length of 214 nm. FIG.1 is the resulting peptide map.

In FIG.1, peptide fragments eluted at about 59, 62 and 68 min after initiating the elution are respectively named peptide fragments 1, 2 and 3. These peptide fragments were separately collected and analyzed for amino acid sequence on "MODEL 473A", a protein sequencer commercialized by Perkin-Elmer Corp., Instrument Div., Norwalk, USA, in conventional manner. As a result, it was revealed that the peptide fragments 1 and 2 have the amino acid sequences of SEQ ID NOs:3 and 7, respectively, while the peptide fragment 3 has those of SEQ ID NOs:4 and 5. The comparison of these amino acid sequences with the one of SEQ ID NO:6 revealed that the peptide fragments 1 to 3 correspond to the positions 148-157, 1-13 and 45-58 or 80-96 in the amino acid sequence of SEQ ID NO:6, respectively. These results confirmed that the peptide fragments 1 and 2 correspond to the C- and N-terminal fragments of the protein used for analysis, and the peptide fragment 3 corresponds to an internal fragment of the protein.

It is concluded that the purified protein obtained by the method in Experiment 1 contains the amino acid sequence of SEQ ID NO:6 when totally evaluating these results, the fact as revealed in Experiment 2 that the purified protein has a main protein band at a position corresponding to a molecular weight of about 18,000-19,500 daltons on SDS-PAGE, and the fact that the purified protein is calculated to have a molecular weight of 18,199 daltons from the amino acid sequence of SEQ ID NO:6.

#### Experiment 4

##### Biological activity

###### Experiment 4-1

###### IFN- $\gamma$ production by immunocompetent cell

Blood was sampled from a healthy volunteer by a heparinized syringe and diluted by 2-fold with serum free RPMI 1640 medium (pH 7.4). The diluted blood was overlaid on a ficoll commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by centrifugation to collect lymphocytes. These lymphocytes were washed with RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal bovine serum and suspended in a fresh preparation of the same medium to give a cell density of  $5 \times 10^6$  cells/ml. The cell suspension was distributed to a 96-well microplate in a volume of 0.15 ml/well.

A purified protein obtained by the method in Experiment 1 was diluted with RPMI 1640 (pH 7.4) supplemented with 10 v/v % fetal bovine serum, and the dilution was distributed to the microplate in a volume of 0.05 ml/well. To the microplate was added a fresh preparation of the same buffer either with or without 2.5  $\mu$ g/ml Con A or 50 units/ml of a recombinant human interleukin 2 in a volume of 0.05 ml/well, and the microplate was incubated at 37°C for 24 hours in a 5 v/v % CO<sub>2</sub> incubator. After completion of the culture, 0.1 ml of a culture supernatant was sampled from each well and assayed for IFN- $\gamma$  activity by conventional enzyme immunoassay (EIA). As a control, a system free of the purified protein was provided and treated similarly as above. The results were in Table 1 where the IFN- $\gamma$  content was assayed and expressed in terms of international unit (IU) with respect to "Gg23-901-530", an international standard for IFN- $\gamma$  obtained from the National Institute for Health, Bethesda, MD, USA.

45

50

55

Table 1

Protein concentration (ng/ml)	IFN-γ yield (IU/ml)		
	Protein	Protein plus Con A	Protein plus interleukin 2
0	<0.5	<2	<0.5
0.32	<0.5	6±2	2±1
1.6	10±2	70±20	60±20
8	140±10	490±80	570±30
40	180±20	620±10	880±50
200	260±20	800±20	1500±400

Note : In the table, the wording "protein" means the present protein.

The results in Table 1 show that lymphocytes as an immunocompetent cell produced IFN- $\gamma$  by the action of the present protein. As is evident from the results, the IFN- $\gamma$  production is increased in the presence of interleukin 2 or Con A as a cofactor.

5    Experiment 4-2

Increase of cytotoxicity by NK cell

10    Blood was sampled from a healthy volunteer by a heparinized syringe and diluted with PBS by 2-fold. The dilution was overlaid on a ficoll, and the resultant was centrifuged to obtain a high density layer of lymphocytes. The lymphocytes were suspended in RPMI 1640 medium (pH 7.2) containing 10  $\mu$ g/ml kanamycin,  $5 \times 10^{-5}$  M 2-mercaptoethanol and 10 v/v fetal bovine serum, and the suspension was distributed to a 12-well microplate in a volume of 0.5 ml/well. A purified protein obtained by the method in Experiment 1 was appropriately diluted with a fresh preparation of the same buffer, and the dilution was distributed to the microplate in a volume of 1.5 ml/well, followed by adding to the microplate 0.5 ml/well of a fresh preparation of the same buffer either with or without 50 units/ml of a recombinant human interleukin 2, incubating the microplate at 37°C for 24 hours in a 5 v/v % CO<sub>2</sub> incubator, and washing the resultant cells with PBS to obtain cultured lymphocytes containing NK cells as an effector cell. 1x10<sup>4</sup> cells/well aliquots of K-562 cells (ATCC CCL243), derived from human chronic myelocytic leukemia as a NK-cell susceptive target cell, which had been labelled with <sup>51</sup>Cr in conventional manner, were distributed to a 96-well microplate, and mixed with the above NK cells in a ratio of 2.5:1, 5:1 or 10:1 (= (effector cells):(target cells)). The microplate was incubated at 37° C for 4 hours in a 5 v/v % CO<sub>2</sub> incubator, followed by counting the radio activity of each supernatant to count the dead target cells. In each system, the percentage (%) of the dead target cells with respect to the target cells used in this experiment was calculated for evaluating cytotoxicity. The results were in Table 2.

25

30

35

40

45

50

55

Table 2

Protein concentration (pM)	Concentration of interleukin 2	Cytotoxicity		
		Effector cells/Target cells 2. 5/1	5/1	10/1
0	0	19	36	59
0	10	28	44	61
0.5	0	22	41	63
0.5	10	31	54	69
5	0	28	49	66
5	10	36	58	71
50	0	29	53	67
50	10	42	62	72
500	0	33	56	84
500	10	57	78	96

Note : In the table, the symbol "pM" means  $10^{-12}$  M, and the wording "protein" means the present protein.

The results in Table 2 show that the protein according to the present invention has a property of enhancing the cytotoxicity by NK cells. As is evident from the results, the cytotoxicity is more enhanced by the coexisting interleukin 2.

Experiment 4-3

5

Induction of LAK cell formation

10

$1 \times 10^4$  cells/well aliquots of Raji cell (ATCC CCL86), a human Burkitt's lymphoma as an NK-cell non-susceptive target cell labelled with  $^{51}\text{Cr}$  in conventional manner were distributed to a 96-well microplate, and mixed with a cell suspension of the target cells and cultured lymphocytes containing LAK cells as an effector cell, prepared similarly by the method in Experiment 4-2 except for culturing 72 hours, in a ratio of 5:1, 10:1 or 20:1 (=effector cells):(target cells)), followed by incubating the microplate at 37°C for 4 hours in a 5 v/v %  $\text{CO}_2$  incubator and counting the radio activity of each supernatant in conventional manner. Thereafter, the cytotoxicity (%) was calculated similarly as in Experiment 4-2. The results were in Table 3.

15

20

25

30

35

40

45

50

55

Table 3

Protein concentration (pM)	Concentration of interleukin 2 (pM)	Cytotoxicity		
		Effector cells/Target cells 5/1	10/1	20/1
0	0	12	23	31
0	10	14	25	35
0.5	0	14	24	34
0.5	10	18	32	42
5	0	16	26	37
5	10	21	36	50
50	0	22	41	49
50	10	26	52	56
500	0	27	44	61
500	10	33	59	72

Note : In the table, the symbol "pM" means  $10^{-12} M$ , and the wording "protein" means the present protein.

The results in Table 3 show that the present protein has a property of inducing the LAK-cell formation. As is evident from these results, this induction is more enhanced by the coexisting interleukin 2.

Experiment 5

5

Acute toxicity test

A purified protein obtained by the method in Experiment 1 was injected percutaneously, orally or intraperitoneally into 8-week-old mice in conventional manner. As a result, the LD<sub>50</sub> of the protein was about one mg/kg mouse or higher independent of these administration routes. This evidences that the present protein is safe to incorporate into medicaments which are administrable to humans.

10 It is well known that IFN- $\gamma$  deeply relates to the inhibition of bacterial infection and the propagation of malignant tumors, the regulation of human biophylaxis through the immunoregulatory function, and to the inhibition of immunoglobulin E antibody's production. As is described above, IFN- $\gamma$  is now commercially available and used as an agent for human susceptive diseases, and the diseases to be treated, dose, administration, and safety are almost revealed. It is described in "Cytokines in Cancer Therapy", edited by Frances R. Balkwill, translated by Yoshihiko WATANABE, published by Tokyo-Kagaku-Dojin, Tokyo, Japan (1991) that treatments using killer cells such as NK- and LAK-cells are used as an antitumor immunotherapy and applied to human diseases, and reported that most of them exert a satisfactory therapeutic effect. Recently focussed is the relationship between the therapeutic effect and the augmentation of killer cells' cytotoxicity or the induction of killer cells' formation using cytokines. For example, T. Fujioka et al. reported in "British Journal of Urology", Vol. 73, No. 1, pp.23-31 (1994) that interleukin 2 strongly induced the formation of LAK cells in an antitumor immunotherapy using LAK cells and interleukin 2, and exerted a satisfactory effect on the metastasis of human cancer without substantially inducing serious toxicity and side effects.

15 Thus it is revealed that IFN- $\gamma$  and killer cells closely relate to the treatment and the prevention of human diseases for complete cure and remission. Under these backgrounds as shown in the results in Experiments 4 and 5, the fact that the present protein induces the IFN- $\gamma$  production by immunocompetent cells, enhances the NK cells' cytotoxicity, and induces the LAK cells' formation indicates that the present agent containing the protein can be administered to humans over a relatively long period of time and exerts a satisfactory therapeutic effect on the treatment and the prevention of IFN- $\gamma$  and/or killer cell related diseases without substantially inducing serious side effects.

20 25 30 The following Examples explain the preferred embodiments of the present invention in more detail. Examples A-1 to A-8 are the preferred embodiments of the preparation of the present protein, and Examples B-1 to B-6 are the preferred embodiments of the present agent for susceptive diseases:

Example A-1

35

Preparation of protein

New born hamsters were suppressed their immunoreaction in conventional manner by injecting a rabbit antiserum to hamster antithymus into the hamsters, transplanted to their dorsal subcutaneous tissues with about 5x10<sup>5</sup> cells/40 hamster of THP-1 cells (ATCC TIB202), a myelomonocytic cell line of a human acute leukemia, and fed for 3 weeks in conventional manner. Tumor masses, about 15 g weight each, subcutaneously formed in each hamster were extracted, suspended in physiological saline in conventional manner, and washed with PBS.

40 45 50 In accordance with the method by Matthew J. Kostura et al. in "Proceedings of the National Academy of Sciences of the United States of America", Vol. 86, pp.5,227-5,231 (1989), the suspended cells were washed with 10-fold volumes of cold 20 mM Hepes buffer (pH 7.4) containing 10 mM potassium chloride, 1.5 mM magnesium chloride, 0.1 mM disodium ethylenediaminetetraacetate, allowed to stand in 3-fold volumes of a fresh preparation of the same buffer, allowed to stand for 20 min under ice-chilled conditions, lyophilized at -80°C, and thawed to disrupt cells. The disrupted cells were centrifuged, and the supernatant was fed to a column packed with "DEAE-SEPHAROSE", a gel for ion-exchange chromatography commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by washing the column with 10 mM phosphate buffer (pH 6.6), fed with a gradient buffer of sodium chloride increasing stepwisely from 0 M to 0.5 M, and collecting fractions eluted at about 0.2 M sodium chloride.

55 The fractions were pooled, dialyzed against 10 mM phosphate buffer (pH 6.8), fed to a column packed with "DEAE 5PW", a gel for ion-exchange chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM phosphate buffer (pH 6.8), fed with a linear gradient buffer of sodium chloride increasing from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.8), and collected fractions eluted at about 0.2-0.3 M sodium chloride.

The resulting fractions were pooled and dialyzed against PBS. The dialyzed inner solution was fed to a cylindrical plastic column prepared by first packing a gel for immunoaffinity chromatography of a monoclonal antibody, which had

been prepared according to the method disclosed in Japanese Patent Application No.58,240/95 applied by the present applicant, then washing with PBS. One hundred mM glycine-HCl buffer (pH 2.5) was fed to the column to effect fractionation, followed by collecting fractions containing a protein which induces the IFN- $\gamma$  production by immunocompetent cells from the eluate, dialyzing the fractions against sterile distilled water, concentrating the dialyzed inner solution with a membrane filter, and lyophilizing the concentrate to obtain a solid purified protein. The yield was about 50 ng per hamster.

#### Example A-2

##### Preparation of protein

New born nude mice were injected into their dorsal subcutaneous tissues with about  $1 \times 10^6$  cells/nude mouse of KG-1 cells (ATCC CCL246), a myelomonocytic cell line derived from human acute myelomonocytic leukemia, and fed for 4 weeks in conventional manner. Tumor masses, about 20 g weight each, formed subcutaneously in each nude mouse were extracted and dispersed in physiological saline in conventional manner. The cells were washed and disrupted similarly as in Example A-1, and the resulting mixture was purified to obtain a purified protein which induces the IFN- $\gamma$  production by immunocompetent cells in a yield of about 20 ng per nude mouse.

A portion of the purified protein was analyzed for amino acid sequence in accordance with the method in Experiments 2-4, revealing that the protein has the partial amino acid sequence of SEQ ID NO:1 near at the N-terminus and a similar molecular weight and biological activity as the protein in Experiment 1.

#### Example A-3

##### Preparation of protein

HL-60 cells (ATCC CCL240), a myelomonocytic cell line derived from human acute promyelocytic leukemia, were suspended in RPMI 1640 (pH 7.4) placed in an about 10-ml plastic cylindrical diffusion chamber in which was installed a membrane filter with a diameter of 0.5  $\mu$ m, then the chamber was intraperitoneally embedded in an aged rat. The rat was fed for 4 weeks in conventional manner, then the chamber was removed. The propagated cells in the chamber were collected, washed with physiological saline, and disrupted similarly as in Example A-1, followed by purifying the resulting mixture to obtain a purified protein which induces the IFN- $\gamma$  production by immunocompetent cells. The yield was about 20 ng per rat.

A portion of the purified protein was analyzed for amino acid sequence in accordance with the method in Experiments 2-4, revealing that the protein has the partial amino acid sequence of SEQ ID NO:1 near at the N-terminus and has a similar molecular weight and biological activity to the protein in Experiment 1.

#### Example A-4

##### Preparation of protein

THP-1 cells (ATCC TIB202), a myelomonocytic cell line derived from human acute monocytic leukemia, were suspended in RPMI 1640 medium (pH 7.2) supplemented with 10 v/v % fetal bovine serum to give a cell density of about  $3 \times 10^5$  cells/ml, and cultured at 37°C for 3 weeks in a 10 v/v % CO<sub>2</sub> incubator while replacing the medium with a fresh one. The propagated cells were separated from the resulting culture, washed with physiological saline, and disrupted similarly as in Example A-1, followed by purifying the resulting mixture to obtain a purified protein which induces the IFN- $\gamma$  production in a yield of about 10 ng per litter of the culture.

A portion of the purified protein was analyzed for amino acid sequence in accordance with the method in Experiments 2-4, revealing that the protein has the partial amino acid sequence of SEQ ID NO:1 near at the N-terminus and has a similar molecular weight and biological activity to the protein in Experiment 1.

50

#### Example A-5

##### Preparation of protein

55 New born hamsters were immunosuppressed by injecting a rabbit antithymus serum in conventional manner, injected to the dosal subcutaneous tissues with about  $5 \times 10^5$  cells/head of A-253 cells (ATCC HTB41), an epidermoid carcinoma, submaxillary gland, human, and fed for 3 weeks in usual manner. Thereafter, the tumor masses formed subcutaneously, about 10 g weight in each hamster, were extracted, dispersed in physiological saline, and washed

with PBS.

The propagated cells thus obtained were washed with 20 mM Hepes buffer (pH 7.4) containing 10 mM potassium chloride, 1.5 mM magnesium chloride, and 0.1 mM disodium ethylenediaminetetraacetate, suspended in a fresh preparation of the same buffer to give a cell density of about  $2 \times 10^7$  cells/ml, disrupted by a homogenizer, and centrifuged to remove cell debris to obtain a supernatant, followed by concentrating the supernatant by a membrane for ultrafiltration to obtain a cell extract containing a protein which induces the interferon- $\gamma$  production by immunocompetent cells. The extract was purified similarly as the method in Example A-1, concentrated, and lyophilized to obtain a solid purified protein in a yield of about 3  $\mu$ g of per hamster.

The purified protein was sampled and analyzed in accordance with the methods in Examples 2-4 revealing that it has the amino acid sequence of SEQ ID NO:1 nearness to the N-terminus and has a similar molecular weight and biological activities to those of the protein in Experiment 1.

#### Example A-6

##### 15 Preparation of protein

A seed culture of A-253 cell was inoculated into RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum and cultured in conventional manner at 37°C until forming a monolayer of cells. Thereafter, the cells were detached from the surface of the culture vessel used by using "TRYPSIN-EDTA", a trypsin commercialized by Giboco BRL, NY, USA, and washed with PBS. In accordance with the method in Example A-1, the cells were disrupted, and the disrupted cells were purified and centrifuged to obtain a supernatant which was then incubated at 37°C for 6 hours, purified, concentrated, and lyophilized to obtain a solid purified protein which induces the IFN- $\gamma$  production by immunocompetent cells in a yield of about one  $\mu$ g per  $10^7$  cells.

The supernatant was sampled and analyzed in accordance with the method in Experiments 2-4 revealing that it has the amino acid sequence of SEQ ID NO:1 near at the N-terminus and has a similar molecular weight and biological activities to those of the protein in Experiment 1.

#### Example A-7

##### 30 Preparation of protein

A seed culture of A-253 cell was inoculated into RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum and cultured in conventional manner at 37°C until forming a monolayer of cells. Thereafter, the culture medium was replaced with a serum-free RPMI 1640 medium (pH 7.4) supplemented with 10 IU/ml of a natural IFN- $\gamma$  derived from KG-1 cell as an IFN- $\gamma$  inducer, and incubated at 37°C for 48 hours. The culture was centrifuged to obtain a supernatant which was then purified by the method in Example A-1, concentrated, and lyophilized to obtain a solid purified protein which induces the IFN- $\gamma$  production by immunocompetent cells in a yield of about 5 ng per  $10^7$  cells.

The supernatant was sampled and analyzed in accordance with the method in Experiments 2-4 revealing that it has the amino acid sequence of SEQ ID NO:1 nearness to the N-terminus and has a similar molecular weight and biological activities to those of the protein in Experiment 1.

#### Example A-8

##### 45 Preparation of protein

A purified protein obtained by the method in Example A-1 was dissolved in an adequate amount of sterile distilled water, and the solution was fed to a column packed with "ASAHPAK® C4P-50 4E", a gel for high-performance liquid chromatography commercialized by Showa Denko K.K., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetic acid, followed by washing the column with 0.1 v/v % aqueous trifluoroacetic acid and feeding to the column a linear gradient solution of acetonitrile increasing from 0 v/v % to 90 v/v % in a mixture solution of trifluoroacetic acid and acetonitrile at a flow rate of 60 ml/hour. Fractions containing a protein which induces the IFN- $\gamma$  production by immunocompetent cells were collected from the eluted fractions, pooled, neutralized with 1 M aqueous tris solution (pH 11.2), and concentrated in conventional manner, followed by removing acetonitrile from the resulting concentrate to obtain a concentrated protein with a purity of at least 95% in a yield of about 10% by weight with respect to the material protein, d.s.b.

In accordance with the method in Experiment 2, the concentrated protein was sampled and analyzed for molecular weight, resulting in a single protein band, which induces an IFN- $\gamma$  production, at a position corresponding to a molecular weight of  $18,400 \pm 1,000$  daltons. Another fresh sample was analyzed for amino acid sequence in accordance with the

method in Experiments 3 and 4, revealing that it has the amino acid sequence of SEQ ID NO:3 and the one of SEQ ID NO:1 near at the N-terminus, more particularly, the one of SEQ ID NO:7, and further it has the amino acid sequence of SEQ ID NOs:4 and 5 as an internal fragment and exhibited a similar biological activity to the protein of Experiment 1 even when concentrated into a relatively high level.

5

#### Example B-1

##### Liquid

10 A purified protein obtained by the method in Example A-1 was dissolved in physiological saline containing one w/v % human serum albumin as a stabilizer, followed by steriley filtering the solution to obtain a liquid.

The product with a satisfactory stability can be used as an injection, collunarium or nebula to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

15

#### Example B-2

##### Dried injection

20 A purified protein obtained by the method in Example A-2 was dissolved in physiological saline containing one w/v % of a purified gelatin as a stabilizer, and the solution was steriley filtered in conventional manner. The sterile solution was distributed to vials by one ml and lyophilized, then the vials were cap sealed.

The product with a satisfactory stability can be used as an injection, collunarium or nebula to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

25

#### Example B-3

##### Dry injection

30 A solid pharmaceutical was prepared similarly as in Example B-2 except for using a purified protein obtained by the method in Example A-5 and "TREHAOSE", a crystalline trehalose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan, as a stabilizer.

The product with a satisfactorily stability can be advantageously used as a dry injection for treating and/or preventing malignant tumors, viral diseases, bacterial infections, and immunopathies.

35

#### Example B-4

##### Ointment

40 "HI-BIS-WAKO 104", a carboxyvinylpolymer commercialized by Wako Pure Chemicals, Tokyo, Japan, and "TRE-HAOSE", a crystalline trehalose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan, were dissolved in sterile distilled water in respective amounts of 1.4 w/w % and 2.0 w/w %, and the solution was mixed to homogeneity with a purified protein obtained by the method in Example A-3, then adjusted to pH 7.2 to obtain a paste containing about one mg of a purified protein per g of the paste.

45 The product with a satisfactory spreadability and stability can be used as an injection, collunarium or nebula to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

#### Example B-5

##### Tablet

50 A purified protein obtained by the method in Example A-4 and "LUMIN (1-1'-1"-triheptyl-11-chinolyl(4)-4,4'-pentamethinchynocyanine-1-1"-dijodide)" as a cell activator were mixed to homogeneity with "FINETOSE®", an anhydrous crystalline  $\alpha$ -maltose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan, and the mixture was tabletted in conventional manner to obtain tablets, about 200 mg weight each, containing the purified protein and LUMIN in an amount of one mg each.

The product with a satisfactory swallowability, stability and cell-activating activity can be used as an injection, collunarium or nebula to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, microbism,

and immunopathies.

Example B-6

5 Agent for adoptive immunotherapy

Human monocytes were separated from peripheral blood of a patient with malignant lymphoma, suspended in RPMI 1640 medium (pH 7.2), which had been supplemented with 10 v/v % human AB serum and preheated at 37°C, to give a cell density of about  $1 \times 10^6$  cells/ml, mixed with about 10 ng/ml of a purified protein obtained by the method in Example A-1 and about 100 units/ml of a recombinant human interleukin 2, and incubated at 37°C for one week, followed by centrifugally collecting LAK cells.

The L'AK cells exerted a strong cytotoxicity on lymphoma cells when introduced into the patient, and the therapeutic effect is significantly higher than that of the conventional adoptive immunotherapy using interleukin 2 alone. Cytotoxic T-cells, obtained by treating a patient's tumor tissue invasive lymphocyte instead of the patient's monocytes, showed a similar effect as in the LAK cells when reintroduced into the patient. The agent for adoptive immunotherapy can be suitably applied to solid tumors such as malignant nephroma, malignant melanoma, large intestinal cancer, and lung cancer.

As is described above, the present invention was made based on a novel protein which induces the IFN- $\gamma$  production by immunocompetent cells and a discovery of human cells which produce the protein. The protein with a partly revealed amino acid sequence stably induces the IFN- $\gamma$  production by immunocompetent cells. Therefore, the protein can be used widely as an IFN- $\gamma$  inducer for IFN- $\gamma$  production by culturing cells, and a therapeutic and/or prophylactic agent for IFN- $\gamma$  susceptible diseases such as viral diseases, malignant tumors, and immunopathies which are susceptible to IFN- $\gamma$ . The present agent for susceptive diseases which contains the protein as an effective ingredient exerts an outstanding effect on the treatment of inveterate diseases such as malignant tumors.

Because the protein has a strong IFN- $\gamma$  production inducibility and has a relatively low toxicity, it induces generally a desired level of IFN- $\gamma$  production with only a small amount and does not substantially cause serious side effects even when administered to patients at a relatively high dose. Therefore, the protein is advantageous in that it quickly induces a desired level of IFN- $\gamma$  production without strictly controlling the dose. Especially, the present protein of human cell origin is advantageous in that it less causes side effects and less induces antibodies when administered to humans in the form of a pharmaceutical composition as compared with artificially produced polypeptides by the recombinant techniques.

The present protein having these satisfactory properties can be produced in a desired amount by the present process using human cells.

Thus the present invention with these significant functions and effects is a significant invention which greatly contributes to this field.

While there has been described what is at present considered to be the preferred embodiments of the invention, it will be understood the various modifications may be made therein, and it is intended to cover in the appended claims all such modifications as fall within the true spirit and scope of the invention.

40

45

50

55

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

NAME:KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU  
KENKYUJO

(ii) TITLE OF INVENTION: PROTEIN WHICH INDUCES INTERFERON- $\gamma$   
PRODUCTION BY IMMUNOCOMPETENT CELL

(iii) NUMBER OF SEQUENCES: 10

## (iv) ADDRESS:

(A) ADDRESSEE:KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU  
KAGAKU KENKYUJO  
(B) STREET: 2-3, 1-CHOME, SHIMOISHII  
(C) CITY:OKAYAMA  
(E) COUNTRY:JAPAN  
(F) POSTAL CODE (ZIP):700

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:Floppy disk  
(B) COMPUTER:IBM PC compatible  
(C) OPERATING SYSTEM:PC-DOS/MS-DOS  
(D) SOFTWARE:Word Perfect Version 5.0

## (vi) PRIOR APPLICATION DATA:

(A1) APPLICATION NUMBER:JP 270,725/95  
(B1) FILING DATE:September 26, 1995  
(A2) APPLICATION NUMBER:JP 67,434/96  
(B2) FILING DATE:February 29, 1996  
(A3) APPLICATION REFERENCE NUMBER:10050403  
(B3) FILING DATE:September 20, 1996

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:10 amino acids  
(B) TYPE:amino acid  
(D) TOPOLOGY:linear

## (ii) MOLECULE TYPE:peptide

## (v) FRAGMENT TYPE:N-terminal fragment

## (xi) SEQUENCE DESCRIPTION:SEQ ID NO:1:

SEQ ID NO:1:

Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser  
1 5 10

## (3) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 5 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: N-terminal fragment  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

SEQ ID NO: 2:

10 Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn  
 1 5 10 15  
 Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp  
 20 25 30  
 15 Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile  
 35 40 45  
 Ile Ser  
 50

20 (4) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 10 amino acids  
 (B) TYPE: amino acid  
 25 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: C-terminal fragment  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

30 SEQ ID NO: 3

Ser Ile Met Phe Thr Val Gln Asn Glu Asp  
 1 5 10

35 (5) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: amino acid  
 40 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal fragment  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

45 SEQ ID NO: 4

Thr Ile Phe Ile Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg  
 1 5 10

50 (6) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 17 amino acids  
 55 (B) TYPE: amino acid

(D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 (v)FRAGMENT TYPE:internal fragment  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:5:

5

SEQ ID NO:5

10	Ile Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr
	1 5 10 15
	Lys

15

(7) INFORMATION FOR SEQ ID NO:6:

20

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:157 amino acids  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear

(ii)MOLECULE TYPE:protein

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:6:

SEQ ID NO:6

25	Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
	1 5 10 15
	Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
	20 25 30
	Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
	35 40 45
30	Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
	50 55 60
	Ser Val Lys Cys Glu Lys Ile Ser Xaa Leu Ser Cys Glu Asn Lys Ile
	65 70 75 80
35	Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
	85 90 95
	Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
	100 105 110
	Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
	115 120 125
40	Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
	130 135 140
	Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
	145 150 155

45

(8) INFORMATION FOR SEQ ID NO:7:

50

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:13 amino acids  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide

(v)FRAGMENT TYPE:internal fragment

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:7:

55

SEQ ID NO:7

Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg  
 1                   5                   10

5

(9) INFORMATION FOR SEQ ID NO:8:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:25 amino acids  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 (v)FRAGMENT TYPE:internal fragment  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:8:

15

SEQ ID NO:8

Ile Ile Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile  
 1                 5                 10                 15  
 Gln Ser Asp Leu Ile Phe Phe Gln Lys  
 20                 25

25 (10)INFORMATION FOR SEQ ID NO:9:

(i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:18  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 (v)FRAGMENT TYPE:internal fragment  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:9:

35 SEQ ID NO:9

Gln Pro Val Phe Glu Asp Met Thr Asp Ile Asp Gln Ser Ala Ser Glu  
 1                 5                 10                 15  
 Pro Gln

40

(11)INFORMATION FOR SEQ ID NO:10:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:471 base pairs  
 (B)TYPE:nucleic acid  
 (C)strandedness:double  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:cDNA to mRNA  
 (vi)ORIGINAL SOURCE:  
 (A)ORGANISM:mouse  
 (B)INDIVIDUAL ISOLATE:liver  
 (ix)FEATURE:  
 (A)NAME/KEY:mat peptide  
 (B)LOCATION:1..471  
 (C)IDENTIFICATION METHOD:S  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:10:

SEQ ID NO:10

5 AAC TTT GGC CGA CTT CAC TGT ACA ACC GCA GTA ATA CGG AAT ATA AAT 48  
 Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn  
 1 5 10 15  
 GAC CAA GTT CTC TTC GTT GAC AAA AGA CAG CCT GTG TTC GAG GAT ATG 96  
 Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met  
 10 20 25 30  
 ACT GAT ATT GAT CAA AGT GCC AGT GAA CCC CAG ACC AGA CTG ATA ATA 144  
 Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile  
 15 35 40 45  
 TAC ATG TAC AAA GAC AGT GAA GTA AGA GGA CTG GCT GTG ACC CTC TCT 192  
 Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser  
 20 50 55 60  
 GTG AAG GAT AGT AAA AYG TCT ACC CTC TCC TGT AAG AAC AAG ATC ATT 240  
 Val Lys Asp Ser Lys Xaa Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile  
 25 65 70 75 80  
 TCC TTT GAG GAA ATG GAT CCA CCT GAA AAT ATT CAT GAT ATA CAA AGT 288  
 Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser  
 30 85 90 95  
 GAT CTC ATA TTC TTT CAG AAA CGT GTT CCA GGA CAC AAC AAG ATG GAG 336  
 Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu  
 35 100 105 110  
 TTT GAA TCT TCA CTG TAT GAA GGA CAC TTT CTT GCT TGC CAA AAG GAA 384  
 Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu  
 40 115 120 125  
 GAT GAT GCT TTC AAA CTC ATT CTG AAA AAA AAG GAT GAA AAT GGG GAT 432  
 Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Asp Glu Asn Gly Asp  
 45 130 135 140  
 30 AAA TCT GTA ATG TTC ACT CTC ACT AAC TTA CAT CAA AGT 471  
 Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser  
 45 145 150 155

35

## Claims

1. A protein of human cell origin, which has the amino acid sequence of SEQ ID NO:1 near or at the N-terminus and induces the interferon- $\gamma$  production by an immunocompetent cell.
2. A protein according to claim 1, wherein one or more amino acids in SEQ ID NO:1 are replaced with other amino acid(s), or one or more amino acids are added to the amino acid sequence of SEQ ID NO:1 without altering the property of the protein.
3. A protein according to claim 1 or 2, which has the amino acid sequence of SEQ ID NO:2 at the N-terminus.
4. A protein according to claim 3, wherein one or more amino acids in SEQ ID NO:2 are replaced with other amino acid(s), or one or more amino acids are added to the amino acid sequence of SEQ ID NO:2 without altering the property of the protein.
5. A protein according to any one of the preceding claims, which has the amino acid sequence of SEQ ID NO:3 near or at the C-terminus.
6. A protein according to claim 5, wherein one or more amino acids in SEQ ID NO:3 are replaced with other amino acid(s), or one or more amino acids are added to the amino acid sequence of SEQ ID NO:3 without altering the property of the protein.

7. A protein according to any one of the preceding claims, which has the amino acid sequences of SEQ ID Nos: 4 and 5 as internal fragments.

5 8. A protein according to any one of the preceding claims, which has the amino acid sequence of SEQ ID NO:6 where the symbol "Xaa" is "isoleucine" or "threonine".

9. A protein according to any one of the preceding claims, which has a molecular weight of 14,000-24,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

10 10. A protein according to any one of the preceding claims, which enhances the cytotoxicity of and induces the formation of killer cells.

11. A protein according to any one of the preceding claims, which is derived from a human hematopoietic cell.

15 12. A process for producing a protein as defined in any one of the preceding claims, which comprises propagating a human cell which produces the protein, and collecting the produced protein from the propagated cells.

13. A process according to claim 12, wherein the human cell is a human hematopoietic cell.

20 14. A process according to claim 12 or 13, which comprises transplanting the human cell to a warm-blooded animal excluding human, and propagating the cell while allowing to receive the body fluid of the animal.

15. A process according to claim 14, wherein the animal is a rodent.

25 16. A process according to any one of claims 12 to 15, wherein the propagated cells are disrupted, then the protein is collected from the resulting mixture.

17. A process according to any one of claims 12 to 16, wherein the propagated cells are subjected to the action of an inducer.

30 18. A process according to claim 17, wherein the inducer is a member selected from the group consisting of mitogens and IFN- $\gamma$ s.

19. A process according to any one of claims 12 to 18, wherein the protein is collected by salting out, dialysis, filtration, concentration, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, absorption chromatography, affinity chromatography, chromatofocusing, gel electrophoresis and/or isoelectrophoresis.

35 20. An agent which comprises a protein as defined in any one of claims 1 to 11 as an effective ingredient and a pharmaceutically-acceptable carrier.

40 21. An agent according to claim 20, which additionally contains interleukin 2.

22. An agent according to claim 20 or 21, which contains serum albumin and/or gelatin as a stabilizer.

45 23. An agent according to claim 20, 21 or 22, which is used as an antioncotic agent.

24. An agent according to claim 23, which is used as an agent for antitumor immunotherapy.

50 25. An agent according to claim 20, 21 or 22, which is used as an antiviral agent.

26. An agent according to claim 20, 21 or 22, which is used as an antibacterial agent.

27. An agent according to claim 20, 21 or 22, which is used as an agent for immunopathy.

55 28. An agent according to claim 20, 21 or 22, which is used for treating atopic diseases.

29. An agent according to any one of claims 20 to 28, which further contains interleukin 12.

30. An agent according to any one of claims 20 to 29, which contains 0.000001-100 w/w % of the protein, on a dry solid basis.

5

10

15

20

25

30

35

40

45

50

55

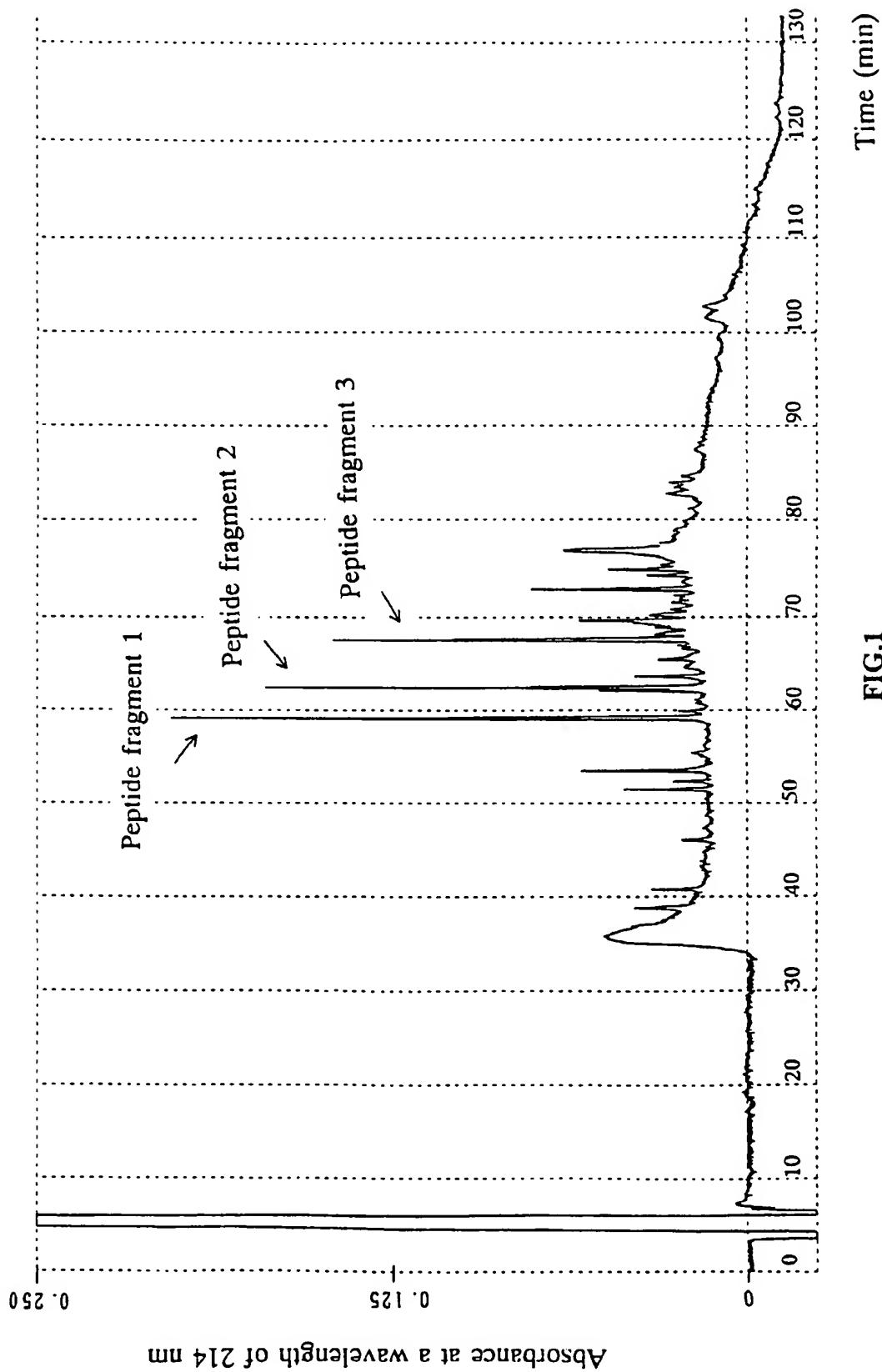


FIG.1



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 96 30 6997

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,P, X	EP 0 712 931 A (HAYASHIBARA BIOCHEM LAB) 22 May 1996 * claims; examples * ---	1-10, 20-30	C07K14/52 A61K38/19 C12P21/02
P,X	EP 0 692 536 A (HAYASHIBARA BIOCHEM LAB) 17 January 1996 * claims; examples * ---	2,4,6,9, 10,20-30	
P,X	JOURNAL OF IMMUNOLOGY, vol. 156, no. 11, 1 June 1996, BALTIMORE US, pages 4274-4279, XP002022861 S. USHIO ET AL.: "Cloning of the cDNA for Human IFN-gamma-Inducing Factor, Expression in Escherichia coli, and Studies on the Biologic Activities of the Protein" * page 4277, right-hand column, paragraph 2 - page 4279, left-hand column, paragraph 3; figures 1-3 * ---	1-10	
D,A	GB 2 016 015 A (HAYASHIBARA CO;ASHIDA S) 19 September 1979 * claims; examples * ---	11-19	
A	WO 92 05256 A (GENETICS INST ;WISTAR INST (US)) 2 April 1992 * the whole document * -----	1-30	
<b>TECHNICAL FIELDS SEARCHED</b> (Int.Cl.6)			
C07K A61K C12P			
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	16 January 1997	Fuhr, C	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons -----           & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**